

REPORT DOCUMENTATION PAGE

AD-A255 054

Public reporting burden for this collection of information is estimated to average 1 hour per response, including gathering and maintaining the data needed, and completing and reviewing the collection of information, including suggestions for reducing this burden, to Washington Headquarters Service, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Project, Washington, DC 20503.



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effort

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 9/2/92		3. REPORT TYPE AND DATES COVERED FINAL 4/1/86 - 10/31/91	
4. TITLE AND SUBTITLE Neurochemical Control of Circadian Rhythms				5. FUNDING NUMBERS G N00014-89-J-1640 PE 61153N PR RR04108 TA 4414013	
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgia State University University Plaza Atlanta, GA 30303				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Office of Naval Research 800 N. Quincy St. Arlington, VA 22217-5000				10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES					
12a. DISTRIBUTION/AVAILABILITY STATEMENT Distribution Unlimited 92 9 10 097		DTIC ELECTE SEP 11 1992 S B D		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) The central aim of this grant was to identify which of the various neurotransmitters found within the suprachiasmatic nucleus (SCN) are involved in synchronizing circadian rhythms with the day-night cycle. Our approach was to determine which SCN neurotransmitters are both influenced by light and capable of shifting the phase of circadian rhythms. We have investigated the possible role of a number of neurotransmitters in circadian control (e.g. vasopressin, GABA, neuropeptide Y). However, our primary focus has been on the circadian functions of vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI) and gastrin releasing peptide (GRP). As the result our work on VIP, PHI and GRP we have developed a working hypothesis of the neurochemical mechanisms underlying the synchronization of circadian rhythms with the day-night cycle. The hypothesis, called the ratio hypothesis, states that light communicated to the SCN via afferent pathways sets the ratio of VIP/PHI to GRP available for release from SCN neurons by altering the cellular levels of VIP/PHI mRNA and/or GRP mRNA, and that the ratio of VIP/PHI to GRP released from SCN neurons in response to light determines how the circadian clock is reset by light.					
14. SUBJECT TERMS Peptides, hypothalamus, colocalization, light, gene expression				15. NUMBER OF PAGES 9	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	
				20. LIMITATION OF ABSTRACT SAR	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

92-25049



387586 1099

PROGRESS REPORT

Localization of VIP and PHI-IR and mRNA within the SCN

Since light is communicated to the SCN only via afferent projections and the two major photic projections to the SCN (i.e. RHT and GHT) terminate in the ventrolateral region of the nucleus, we investigated the neurotransmitters contained within this region. Previous studies had identified heavy concentrations of VIP immunopositive neurons in close association with the terminals of the RHT and GHT. However, since the precursor of VIP was subsequently found to contain another neuropeptide, peptide histidine isoleucine (PHI), we examined whether PHI-IR and VIP/PHI mRNA was distributed in the same fashion as VIP-IR using immunocytochemistry and in situ hybridization. PHI-IR was found in heavy concentrations in a pattern that corresponded closely to that of VIP-IR within the ventrolateral region of the rat SCN. Using a ³⁵S labeled riboprobe that recognized the entire coding sequence of VIP/PHI mRNA, VIP/PHI mRNA was found over neuronal cell bodies within the ventrolateral SCN in a pattern similar to that of VIP- and PHI-IR. **CONCLUSIONS:** VIP- and PHI-IR and mRNA are found within the region of the SCN where major photic afferent pathways terminate.

Effects of light on the levels of VIP and PHI-IR within the SCN

Since VIP/PHI neurons within the SCN appear to receive direct input from the RHT and GHT, the next step was to determine if light influenced VIP- and PHI-IR. Micropunches of the SCN and a control region in the medial area of the cortex rich in VIP- and PHI-IR were removed from rats housed in constant light or constant dark for 20 days. SCN concentrations of VIP- and PHI-IR were significantly higher in rats housed in constant darkness as compared to those housed in constant light. In contrast, no significant differences were observed in VIP- and PHI-IR within the cortex of these same rats. To determine whether the effects of light on VIP and PHI were specific to these peptides, neurotensin (NT) and substance P (SP) levels in the SCN were also compared in rats housed in constant light or constant dark for 20 days. Using the same methods as above, the amounts of NT- and SP-IR within the SCN were found to be unaffected by lighting conditions. **CONCLUSIONS:** Light influences SCN concentrations of VIP- and PHI-IR, but not the SCN concentrations of NT- or SP-, or VIP- and PHI-IR concentrations in cortex. These data indicate that light selectively suppresses, and/or darkness increases VIP and PHI-IR within the SCN.

Light-dark rhythm in VIP/PHI mRNA within the SCN

The finding that light influenced VIP- and PHI-IR within the SCN suggested that the SCN levels of VIP/PHI mRNA might vary over the light-dark cycle. To investigate this hypothesis, rats were housed in LD 14:10 for two weeks and killed either 5 hrs after the onset of light or 2 hrs after the onset of darkness. In situ hybridization of coronal sections through the SCN region (carefully matched along the anterior-posterior plane) of rats killed during light or darkness revealed a striking light-dark difference in the density of hybridization signal. Quantification of the autoradiographs revealed significantly higher levels of VIP/PHI mRNA in the SCN of rats killed during the dark phase than in the SCN of rats killed during the light phase. A second experiment confirmed this light-dark rhythm in VIP/PHI mRNA with solution hybridization. Rats housed in LD 14:10 were killed 5 hrs after lights on or 2 hrs after dark

onset. The SCN and a region in the medial cortex were removed with a micropunch and VIP/PHI mRNA were determined with solution hybridization. VIP/PHI mRNA was three times higher in the SCN of rats killed during the dark than in rats killed during the day. Furthermore, the light-dark rhythm in VIP/PHI mRNA was specific to the SCN since no significant changes were observed in the cortex, suggesting that the SCN rhythm was not induced by "nonspecific" factors such as hormone rhythms. **CONCLUSIONS:** There is a significant light-dark rhythm in VIP/PHI mRNA within the SCN, with higher levels of VIP/PHI mRNA occurring during the dark.

Colocalization of VIP/PHI and GRP within the SCN

At the time our VIP/PHI studies were underway, another laboratory reported that VIP-, PHI- and GRP-IR coexist in a subpopulation of SCN neurons. In view of the potential importance of this finding for our studies, we replicated this finding by demonstrating the colocalization of VIP/PHI and GRP mRNA within the SCN using in situ hybridization. Analysis of adjacent sections labeled separately for VIP/PHI mRNA or GRP mRNA indicated that GRP and VIP/PHI mRNA could be found in the same neuron. Using this approach it was not possible to determine the proportion of VIP/PHI neurons containing GRP because the section thickness (12 μ M) was greater than the average diameter of nuclei in SCN neurons (i.e. $6.51 \pm 0.8 \mu$ M); thus, not all nuclei were contained in both sections. Since the abundance of GRP mRNA is relatively low requiring several weeks of exposure to detect the signal, thinner sections would have compromised the ability to detect GRP mRNA in single cells. Nevertheless, the long exposure time necessary for the detection of GRP mRNA suggested that the mRNA encoding GRP is expressed less abundantly than VIP/PHI mRNA. **CONCLUSIONS:** These data confirm immunological data indicating that VIP, PHI and GRP can be colocalized within SCN neurons and suggest that GRP is colocalized in some, but not all, SCN neurons that produce VIP/PHI.

VIP/PHI and GRP mRNAs have opposite 24 hr rhythms within the SCN of rats housed in 24 hr light-dark cycles

VIP/PHI and GRP are colocalized in the SCN and the levels of VIP/PHI mRNA occur in a 24 hr rhythm within the SCN. These findings raised the question as to whether the levels of GRP mRNA were also rhythmic, and if so whether that rhythm paralleled the rhythm in VIP/PHI mRNA. If the patterns of VIP/PHI and GRP mRNAs were similar it would suggest that these mRNAs could be regulated by common mechanisms, however if the patterns were different it would suggest that VIP/PHI and GRP mRNAs might be differentially regulated. To compare the 24 hr patterns of these mRNAs, the cellular levels of VIP/PHI and GRP mRNA in the SCN were determined with quantitative in situ hybridization in adjacent coronal sections obtained at 4 hr intervals from rats housed in LD 14:10. Quantitative analysis of the autoradiographs obtained throughout the SCN again revealed a statistically significant 24 hr rhythm in VIP/PHI mRNA that peaked during the dark phase, and a significant 24 hr rhythm of GRP mRNA that peaked during the light phase. Analysis of the levels of both mRNAs in sections obtained from the anterior and posterior regions of the SCN revealed that the characteristics of the daily patterns of VIP/PHI and GRP mRNAs varied between the anterior and posterior SCN. The rhythm in VIP/PHI mRNA was more pronounced in the anterior than

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in the posterior SCN, while the rhythm in GRP mRNA was more pronounced in the posterior than in the anterior SCN. While the amplitude of the rhythms in both mRNAs varied from the anterior to the posterior SCN, the patterns of VIP/PHI mRNA compared with those of GRP mRNA were distinctly different in both the anterior and posterior SCN. **CONCLUSIONS:** These data demonstrate that VIP/PHI and GRP mRNA exhibit different 24 hr rhythms in neurons of the SCN, suggesting that the ratio of VIP/PHI to GRP available for release within the SCN changes as a function of the time of day. These data also suggest that the regulation of VIP/PHI and GRP mRNA may be somewhat different in the anterior versus posterior SCN.

VIP/PHI and GRP mRNAs do not have 24 hr rhythms in the SCN of rats housed in continuous illumination

The 'ratio hypothesis' predicts that light communicated to the SCN via afferent pathways induces, or at least influences the 24 hr rhythm of VIP/PHI mRNA that peaks at night, and/or the 24 hr rhythm of GRP mRNA that peaks during the day. If so, the 24 hr rhythms in VIP/PHI and GRP mRNAs should be significantly altered, or completely absent in rats housed in continuous darkness. The following experiments provide further support for this prediction. Rats (N=71) were housed in LD 14:10 for 2 wks followed by continuous darkness for 10 days. Circadian wheelrunning rhythms were monitored in all rats. Groups of rats (N=5-6) were decapitated at 4 hr intervals throughout the circadian cycle (i.e. 10, 6 and 2 hrs before the onset of daily wheelrunning and 2, 6 and 10 hrs after the onset of running). The cellular levels of VIP/PHI and GRP mRNAs within the SCN were then determined by *in situ* hybridization of 12 micron thick coronal sections. There was no statistically significant ($P > 0.05$) change in the levels of either VIP/PHI or GRP mRNA over the circadian cycle. This can be compared to the significant changes in the levels of both VIP/PHI and GRP mRNAs of rats housed in LD cycles. **CONCLUSIONS:** These data demonstrate that the 24 hr patterns of VIP/PHI and GRP mRNAs are absent in rats housed in constant darkness. Comparison of these data with the 24 hr patterns of VIP/PHI and GRP mRNAs in rats housed in light-dark cycles indicates that the light-dark cycle either induces or influences the rhythms of VIP/PHI and GRP mRNA. These data therefore provide strong support for the 'ratio hypothesis'.

Microinjection of a cocktail containing VIP, PHI and GRP into the SCN phase shifts circadian rhythms in a manner that mimics the phase delaying effects of light

Since VIP, PHI and GRP coexist in local circuit neurons within the SCN these peptides are probably coreleased within the nucleus. To investigate the possible circadian functions of the corelease of these peptides a cocktail containing equimolar concentrations of VIP, PHI and GRP (VIP/PHI/GRP) was microinjected into the SCN region. Microinjection of VIP/PHI/GRP produced large delays (i.e. 1.53 ± 0.35 hrs) in the phase of the circadian wheelrunning rhythm of hamsters housed in constant light when administered around the time of the onset of the daily active phase, but not at other times within the circadian cycle. The circadian effects of VIP/PHI/GRP appeared to be restricted to altering the phase of circadian rhythms, since VIP/PHI/GRP microinjection did not significantly alter the free-running circadian period. Comparison of the phase shifting effects of VIP/PHI/GRP with the well known effects of light indicated that VIP/PHI/GRP mimicked the phase delaying, but not the phase advancing effects of light. The phase delaying effects of VIP/PHI/GRP were also found to be dose-dependent

when microinjected in concentrations ranging from 0.3 to 3000 μ M. In an effort to localize the effects of VIP/PHI/GRP to the SCN the effects of VIP/PHI/GRP microinjected into the SCN were compared with the effects of VIP/PHI/GRP microinjected into the cerebroventricular system. VIP/PHI/GRP administered into the lateral ventricle during the 3 hr interval following activity onset in the same dose and injection volume as administered into the SCN produced no phase shifts in circadian rhythms. These data support the view that the phase shifting effects of VIP/PHI/GRP are the result of their influence on SCN neurons. **CONCLUSIONS:** These data demonstrate that the combined administration of VIP, PHI and GRP mimic some of the effects of light on circadian rhythms in a dose-dependent manner, and suggest that the corelease of VIP/PHI/GRP may mediate at least some of the phase shifting effects of light.

VIP, PHI and GRP interact within the SCN in a novel fashion to phase delay circadian rhythms

A series of experiments that were critical in the development of the 'ratio hypothesis' examined whether the phase shifting effects of VIP/PHI/GRP required an interaction among all three peptides, or whether one or two of these peptides were sufficient to produce phase shifts. For the ratio of VIP/PHI to GRP to be critical in determining how the circadian clock is phase shifted all three (or at least VIP or PHI and GRP) peptides must be necessary to produce phase shifts within the SCN. In view of the critical nature of these experiments in the development of the 'ratio hypothesis' we examined the possible interaction among these peptides within the SCN with both in vivo behavioral and in vitro cellular studies.

In vivo behavioral analysis: To determine whether VIP, PHI and GRP interact within the SCN to produce a signal capable of phase shifting circadian rhythms, the phase shifting effects of administration of VIP, PHI or GRP alone, and the coadministration of VIP/PHI, VIP/GRP and PHI/GRP were compared with the coadministration of VIP/PHI/GRP. All microinjections into the SCN were administered during the 3 hrs following the onset of activity and the final concentration of total peptide (150 pmol) given in each injection was identical (e.g. the VIP injections contained 150 pmol of VIP and the VIP/PHI/GRP injections contained 50 pmol of VIP, 50 pmol of PHI and 50 pmol of GRP). A statistically significant difference was observed in the magnitude of the phase shifts produced by the various combinations of the three peptides. Administration of VIP, PHI or GRP alone phase delayed the activity rhythm by 30 min or less. Although coadministration of two of the three peptides produced slightly larger phase delays than when given alone, the phase delays produced by coadministration of two peptides remained at least 50% less than those produced by coadministration of VIP/PHI/GRP.

In vitro cellular analysis: The hypothalamic slice preparation was used to investigate the cellular effects of VIP, PHI and GRP and their coadministration on SCN single unit activity. The spontaneous firing of neurons in the ventrolateral SCN was recorded extracellularly during an 8 hr interval beginning at the phase of the circadian cycle corresponding to 4 hr before the time that locomotor activity normally begins (i.e. circadian time 8-16). Coadministration of VIP/PHI/GRP into the perfusate increased the firing rate of single units by an average of 3.85 ± 0.35 impulses/s in 65% of the 23 neurons examined. In contrast, coadministration of any two of the peptides, or VIP, PHI or GRP alone, in the same final concentration of total peptide (i.e. 10^{-7} M), produced significantly less excitation than did coadministration of VIP/PHI/GRP. The excitation of SCN neurons produced by peptide coadministration was dose-dependent.

CONCLUSIONS: An interaction among VIP, PHI and GRP within the SCN is required for the phase shifting effects of these peptides on circadian rhythms. It is also important to note that these peptides interact in a manner that is different than that previously reported for other colocalized messengers, where one neurotransmitter normally constitutes the primary chemical signal, while the other messenger modifies that signal but has little or no activity alone. Demonstration of this novel form of interaction among colocalized messengers illustrates the potential of these SCN studies as a model system for understanding some of the fundamental properties of colocalized neurotransmitters.

Effectiveness of VIP and GRP antagonists within the SCN

We began studies to verify the ability of the VIP and GRP antagonists to block the effects of the corelease of VIP, PHI and GRP within the SCN. Hamsters were implanted with guide cannula aimed at the SCN and allowed to establish free-running circadian activity rhythms in constant light. Hamsters were microinjected with a saline control or one of the VIP or GRP antagonists followed 5 min later by microinjection of an equimolar concentration of VIP/PHI/GRP (3 μ M). At present two VIP and one GRP antagonist have been examined. These preliminary data suggest that these antagonists are effective in blocking the effects of VIP/PHI/GRP within the SCN. **CONCLUSIONS:** These preliminary data suggest that the VIP and GRP antagonists available for these studies will be effective in blocking the effects of VIP, PHI and GRP coreleased within the SCN.

Single unit response of suprachiasmatic neurons to neuropeptide Y in the hamster

The effect of neuropeptide Y (NPY) on the spontaneous discharge of neurons within the hamster (*Mesocricetus auratus*) suprachiasmatic nucleus (SCN) (N=83) was determined using the hypothalamic slice preparation. The discharge of neurons within the ventral SCN recorded during the day was either excited (6/42), immediately inhibited (17/42), or transiently excited and then inhibited (10/42) by NPY 10^{-7} . During the night, NPY produced excitatory effects in 2/23 neurons, inhibition in 7/23 and excitation followed by inhibition in 4/23. A higher percentage of neurons was found to be unresponsive to NPY during the night than during the day. This difference approached but did not reach statistical significance. In the 18 neurons recorded within the dorsal SCN, NPY had little effect on spontaneous discharge during the day or night. **CONCLUSIONS:** These data indicate that bath application of NPY predominately inhibits the spontaneous discharge of SCN neurons recorded in the hypothalamic slice preparation.

Effects of neuropeptide Y on corticosterone levels and single unit activity in the rat

The following study determined whether neuropeptide Y (NPY) acts within the hypothalamic paraventricular nucleus (PVN) or the suprachiasmatic nucleus (SCN) to alter circulating levels of corticosterone and evaluated the effects of NPY on the single-unit response of PVN and SCN neurons using the hypothalamic slice preparation. Blood levels of corticosterone were determined in groups of rats that received microinjections of NPY or saline (Sal) into the PVN or SCN. NPY injected into the PVN 4 h after light onset resulted in corticosterone levels of 13.15 ± 2.18 (SE) μ g/dl within 1 h, which were significantly higher

than the corticosterone levels of $4.08 \pm 1.78 \mu\text{g/dl}$ seen in rats receiving Sal injections. In contrast, no significant differences were observed in circulating levels of corticosterone between groups of rats 1 or 4 h after NPY or Sal microinjection into the SCN. In the hypothalamic slice, NPY was found to produce primarily inhibitory responses in both SCN and PVN neurons. Forty-nine percent of the SCN units examined were inhibited. In addition, another 20% of the neurons tested in the SCN displayed excitation followed by more sustained inhibition. In the PVN, 45% of the units examined were inhibited by NPY, however, nearly 30% of the remaining neurons were significantly excited by NPY. **CONCLUSIONS:** NPY alters the electrical activity of both SCN and PVN neurons but appears to act only within the PVN to influence circulating levels of corticosterone. These and other data indicate that NPY acts as an important neurochemical messenger within several hypothalamic sites.

Single unit response of neurons within the hamster suprachiasmatic nucleus to GABA and low chloride perfusate during the day and night

Using the in vitro hamster hypothalamic slice preparation, the effects of GABA and 80% chloride (Cl^-) reduced medium on the single unit activity of SCN neurons was investigated. GABA 10^{-4} M produced inhibitory responses in 55% of the 69 SCN neurons examined. No statistically significant day-night difference was observed in either the percentage of SCN units responding to GABA, or in their threshold response. During the day 80% Cl^- reduced medium had an excitatory effect on SCN neurons; however, following the return to normal Cl^- concentrations a transient, but significant inhibition was observed. During the night, 80% Cl^- reduced medium produced an excitatory response similar to that observed during the day, but no inhibition following return to the medium containing normal Cl^- concentrations. Only during the night was Cl^- reduced medium found to initiate activity in a dose-dependent manner in some silent cells. No significant day-night difference in response to 80% Cl^- reduced medium occurred in neurons of the paraventricular nucleus of the hypothalamus. **CONCLUSIONS:** These results indicate that SCN neurons whose activity is mediated by Cl^- may be involved in the control of circadian rhythms.

Effects of GABA and anxiolytics on the single unit discharge of suprachiasmatic neurons in the rat

The effects of gamma-aminobutyric acid (GABA), muscimol, baclofen and the anxiolytics; diazepam (DZP), flurazepam (FZP) and zopiclone on single-unit neural activities in the suprachiasmatic nucleus (SCN) were investigated using the rat hypothalamic slice preparation. Exposure of the slice to GABA 10^{-4} M produced inhibitory responses in 65% of the 49 SCN neurons examined. The threshold concentration of GABA ranged from 10^{-6} to 10^{-4} M. Neurons responsive to GABA were not found to be restricted to a subdivision of the SCN, but were diffusely distributed throughout the nucleus. DZP, FZP and zopiclone produced responses similar to those of GABA. The inhibitory effects of GABA (10^{-3} M) were potentiated by coadministration of DZP (10^{-5} M). Muscimol and baclofen (10^{-7} M to 10^{-4} M) also inhibited SCN neuronal activity in a dose-dependent manner. Bicuculline (10^{-5} M- 10^{-4} M) scarcely affected the baclofen-induced inhibition (1/6) but strongly antagonized the effects of muscimol (6/6), GABA (6/8) and DZP (4/5). **CONCLUSIONS:** These results suggest that the receptors mediating the inhibitory effects of GABA and anxiolytics within the SCN may be GABA_A and/or

GABA_B or GABA-BDZ receptor complex, respectively.

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